IMMOBILIZED CARBOXYPEPTIDASE Y. APPLICATIONS IN PROTEIN CHEMISTRY

Frederick A. LIBERATORE, John E. McISAAC, Jr. and Garfield P. ROYER
Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210, USA

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1. Introduction

Carboxypeptidase Y (CPY), a metal-free exopeptidase found in yeast [1–5], catalyzes the hydrolysis of both peptide and ester substrates. The enzyme contains serine and histidine at the active site and exhibits burst kinetics with *p*-nitrophenyl trimethylacetate [6–8].

CPY is of interest to protein chemists because of its broad specificity. Proline is readily released from peptide linkages. Glycine and aspartic acid are released very slowly. We have demonstrated total hydrolysis of proline-free peptides with mixtures of immobilized enzymes [9]. The bound CPY described in this report has no endopeptidase activity and should be of considerable worth when combined with other enzymes in total hydrolysis of polypeptides which contain proline. We demonstrate herein the utility of the bound enzyme in the sequencing of a polypeptide containing proline.

After many unsuccessful attempts to prepare an immobilized CPY derivative exhibiting good operational stability over a wide pH range, we have found that hexamethylenediamine-Cl-Sepharose 4B (prepared by periodate oxidation and reductive alkylation) is the support of choice. The enzyme conjugate was made with water-soluble carbodiimide. The modified Sepharose prepared by the method described here should be useful as a support for other enzymes and affinity ligands.

2. Experimental

2.1. *Carboxypeptidase Y*

Carboxypeptidase Y (EC 3.4.12.1) was prepared according to Kuhn, et al. [5] from Anheuser-Busch baker's yeast. The enzyme was judged pure on the basis of the following criteria: (1) amino acid analysis; (2) constant specific activity across the peak of the final gel permeation column; and (3) absence of endopeptidase activity. Cross-linked agarose (Cl-Sepharose 4B) was purchased from Pharmacia. All other chemicals were reagent grade. Deionized, doubly glass-distilled water was used throughout.

2.2. *Periodate oxidation of cross-linked agarose*

Cl-Sepharose 4B was washed in a Büchner funnel with 0.1 M acetic acid, 1% w/v $NaHCO_3$, and water (50 ml/ml gel in each case). Oxidation was carried out with 0.01 M $NaIO_4$ (10 ml/ml gel) for 2 h at room temperature with shaking. The gel was then washed with water (25 ml/ml gel).

2.3. *Coupling of hexamethylenediamine.*

A solution of hexamethylenediamine (HMD) which was 0.1 M in 0.1 M borate, pH 9.0, was mixed with oxidized gel (2 ml/ml gel) and stirred at room temperature. At t = 20 and 40 min, $NaBH_4$ (0.25 mg/ml) of gel) was added. Twenty minutes after the second addition of borohydride, the reaction was terminated by washing as described in the preparation prior to oxidation. The Gly-D, L-Nle was attached by essentially the same method. The amine content of the gel was determined by the manual ninhydrin method of Moore [10]. Acid hydrolyses of agarose derivatives containing peptide or protein were performed with 6 N HCl in evacuated, sealed tubes at 110°C for 24 h. Amino acid analyses were performed with a Beckman 116 analyser with Durrum single column methodology. The model peptide was provided by Pierce Chemical Co.

2.4. *CPY immobilization*

HMD-Cl-Sepharose was equilibrated with 1 mM pyridine–Cl buffer pH 4.75. To 50 ml of gel suspension (30 ml settled gel), 5 mg of CPY was added. A total amount of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-*p*-toluenesulfonate (WSCD) of 20 mg/mg protein was added in increments over a period of 1 h at 4°C. At the end of an additional 4 h period the reaction was terminated by the washing procedure used earlier.

2.5. *Enzyme assays*

The esterase activity of bound CPY was determined with a pH stat: 50 ml of 10 mM *N*-α-acetyl-L-tyrosine ethyl ester (ATEE), 25°C ± 0.1, 0.1 M KCl, 2% MeOH, 100 mg damp enzyme conjugate, 0.01 m NaOH as titrant. The peptidase activity was determined with CBZ-Leu-Phe by the ninhydrin method of Moore [10]. For the soluble enzyme we used the following conditions: 0.75 μg/ml of CPY in 5 ml of 1.0 mM substrate (0.5 mM at pH 3.5 and 4.0) 0.05 M acetate, phosphate, or borate-carbonate–KCl buffer for the pH ranges of 3.5–6.0, 6.0–8.0, and 8.0–9.0, respectively; the temperature was 25°C ± 0.1. The bound enzyme was assayed by the same method except that aliquots were withdrawn with a syringe capped with fine nylon net. The reaction volume was 10 ml and contained 50 mg damp gel.

2.6. *Digestion of model peptide*

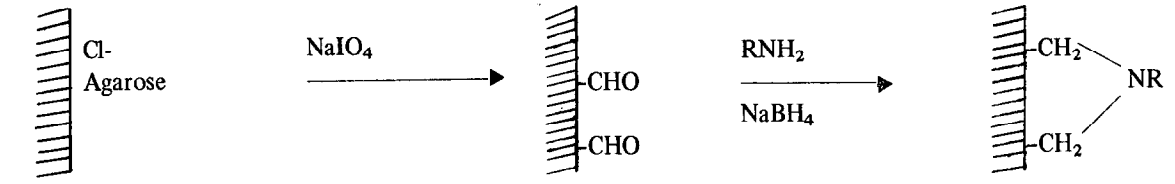
The dodecapeptide, Leu-Gly-Pro-Trp-Val-Arg-Gly-Glu-Ala-Pro-Ile-Lys, was digested by CPY on HMD-Cl-Sepharose using the following conditions: 0.31 mg/ml peptide in 0.1 M *N*-ethylmorpholine acetate, pH 6.0 at 37°C.

3. Results and discussion

3.1. *Preparation of HMD-Cl-Sepharose*

Periodate oxidation of the gel is essentially complete after 2 h. Using longer reaction times or periodate concentrations higher than 0.01 M did not yield a greater amount of HMD bound. After one week of storage at 4°C the oxidized gel bound the same amount of amine as the freshly oxidized material. The pH optimum for coupling is 9.0. This is consistent with previous results for reductive alkylation reactions in related systems [11,12]. It is important to point out that the amine content (140 μmol/g dry gel) of HMD-Cl-agarose is stable after copious washing and use over a broad pH range. This is not the case for alkylamino porous glass and amine derivatives of agarose prepared by the CNBr method [13,14].

Gly-D, L-Nle was coupled by the same technique as for HMD. Hydrolysis, extraction, and amino acid analysis revealed considerable norleucine but little glycine which is consistent with the following scheme.



3.2. *Immobilization of CPY*

Amino acid analyses showed that our procedure yielded 0.2 mg enzyme/100 mg dry agarose. The coupling was 44% efficient. The important point is that this enzyme conjugate is stable over a wide pH range. This is consistent with covalent attachment to the support. The apparent k_{cat} of the bound enzyme was 76 sec^{-1} vs. ATEE which represents a retention of about 70% of the activity after immobilization. The pH optima of the esterase activities of the two enzyme forms are similar (fig.1). The k_{cat} of the bound CPY vs. CBZ-Leu-Phe was 38 sec^{-1} again about 70% of k_{cat} for the soluble enzyme.

The peptidase pH optimum of the bound form is about 0.5 units lower than that of the free form (fig.2). Hydrolysis of the ester results in the production of protons. The hydrolysis of CBZ-Leu-Phe does not result in proton formation. The shift in the pH

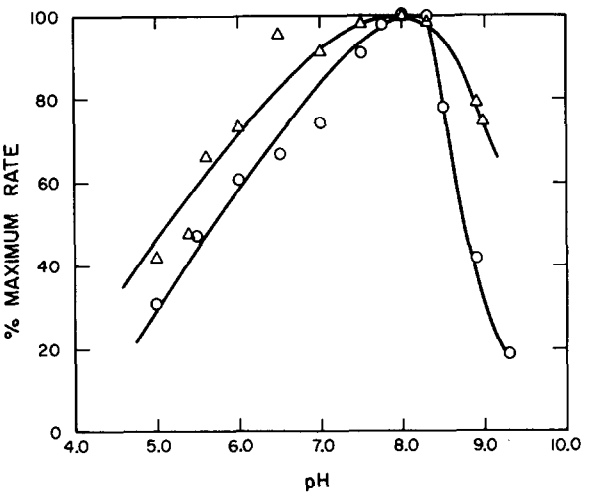


Fig.1. The pH dependencies of esterase activities of soluble CPY (△) [7] and immobilized CPY (○) in 0.1 M KCl with 10 mM ATEE substrate.

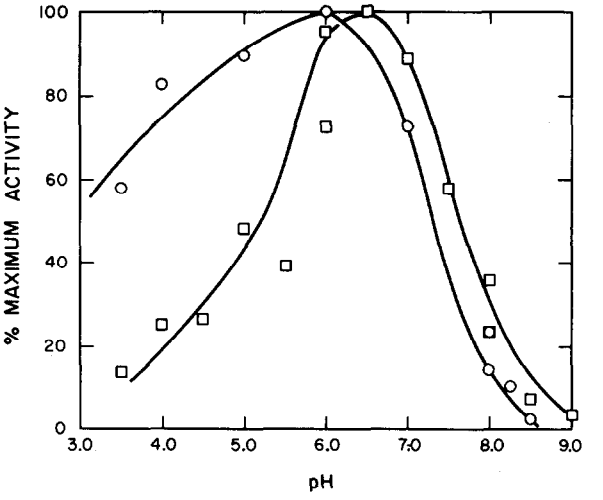


Fig.2. The pH dependencies of peptidase activities of soluble (□) and insoluble CPY (○) against 1.0 mM CBZ-Leu-Phe. Buffers and assay procedure described in the text.

curve for peptidase activity is consistent with a 'micro-environmental' perturbation produced by a positively charged carrier ([15] and references therein). The pH curve for esterase activity of the bound form is shifted slightly to more basic pH. This may be explained by the diffusion-limited efflux of H^+ from the matrix [15].

3.3. *Sequencing of model peptide*

The dodecapeptide, Leu-Gly-Pro-Trp-Val-Arg-Gly-Glu-Ala-Pro-Ile-Lys, was digested by CPY immobilized on HMD-Cl-Sepharose. The data of table 1 demonstrate the utility of our enzyme conjugate in sequence determinations. Although the Arg-Gly bond appears to be hydrolyzed slowly, exopeptidase activity proceeds smoothly through the proline residue.

Acknowledgements

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Table 1
Digestion of a dodecapeptide by CPY immobilized on HMD-Cl-Sepharose

Duration of digestion	Mole percent of release of amino acids											
	Leu	Gly	Pro	Trp	Val	Arg	Gly	Glu	Ala	Pro	Ile	Lys
12 h	*	+	+	**	3.6	1.7	1.1	4.2	8.8	69.8	94.0	89.3
24 h	*	+	+	**	8.6	5.6	2.5	35.5	53.4	89.5	101.0	99.3

*None detected

+All observed Gly and Pro attributed to the residues proximal to the C-terminus.

**Not determined.

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